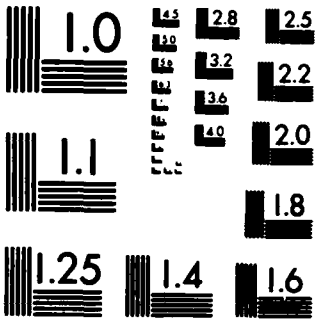

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TESTING OF EXPERIMENTAL COMPOUNDS AGAINST AMERICAN MUCOCUTANEOUS AND CUTANEOUS LEISHMANIASIS

Annual Progress Report

Dr. Jan S. Keithly

February 1981

Supported by

US Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-80-C-0061

Cornell University Medical College
New York, New York 10021

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) TESTING OF EXPERIMENTAL COMPOUNDS AGAINST AMERICAN MUCOCUTANEOUS AND CUTANEOUS LEISHMANIASIS		5. TYPE OF REPORT & PERIOD COVERED Annual July 1980 - January 1981 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Dr. Jan S. Keithly		8. CONTRACT OR GRANT NUMBER(s) DAMD17-80-C-0061
9. PERFORMING ORGANIZATION NAME AND ADDRESS Cornell University Medical College New York, New York 10021		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62770A.3M162770A871.AF.072
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick Frederick, Maryland 21701		12. REPORT DATE February 1981 13. NUMBER OF PAGES 18
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

A. Leishmaniasis

Cutaneous	Mucocutaneous
Leishmania mexicana amazonensis	L. b. braziliensis
L. m. mexicana	L. b. guyanensis
Visceral: L. donovani	L. b. panamensis

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Cutaneous and mucocutaneous Secondary Test Systems

During the first six months of this contract, Leishmania m. mexicana and L. b. panamensis served as standard subspecies for drug screening. Using BALB/c mice, we show that:

1. Both amastigotes and promastigotes yield suitable lesions for drug screening, and that promastigotes grown in insect media are as infective as amastigotes.

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

19. Key Words (continued)

B. Drugs
Difluoromethylornithine
Glucantime
Pentamidine
Pentostam
SD50, SD90
Mode of Action

C. Assays
Amastigotes
Promastigotes
Log
Stationary
Infectivity
Growth
BALB/c mice
J774 macrophages
Intracardial
Intravenous
Schneider's medium
Glycosome

20. Abstract (continued)

1. In Schneider's drosophila medium, subcultured promastigotes remain infective. Stationary phase cells are more infective in all media tested.
2. Time to lesion development is stage and dose dependent.

Using Pentostam and Glucantime to treat developed lesions of L. m. mexicana and L. b. panamensis, we show that:

1. Neither drug can cure these infections.
2. Leishmania b. panamensis ~~is~~ more sensitive to Glucantime
3. The SD50 and 90 for these subspecies ~~is~~ greater than that for visceral L. donovani infections (461 and 800 Sb mkd x 15 and 29 or 58 Sb x 5, respectively).

Visceral Secondary Test Systems

Using L. donovani in BALB/c mice, we show that:

1. Fourteen day assays are highly reliable whether mice are inoculated IC or IV with 10 million splenic amastigotes or promastigotes.
2. Intracardial inoculations will be employed as they allow direct comparison with hamster primary screening test systems.
3. Unlike its effect against bloodstream trypanosomes, the polyamine inhibitor difluoromethylornithine alone given before, at time of, or after infection does not suppress L. donovani infections in BALB/c mice.

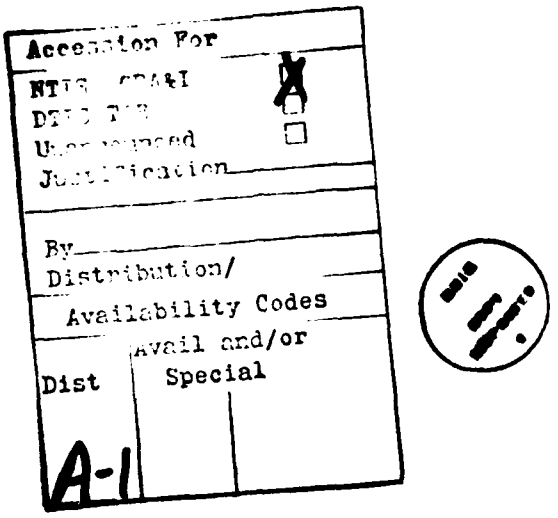
Preliminary ultrastructural evidence (Keithly and Langreth, 1983) indicates that the mode of action of Pentostam may be upon glycolytic enzymes sequ estered within a highly specialized organelle, the glycosome, in leishmania.

A BALB/c macrophage cell line, J774.G.8, supports continuous growth of L. donovani amastigotes in vitro.

FOREWARD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for Care and Use of Laboratory Animals," prepared by the Committees on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



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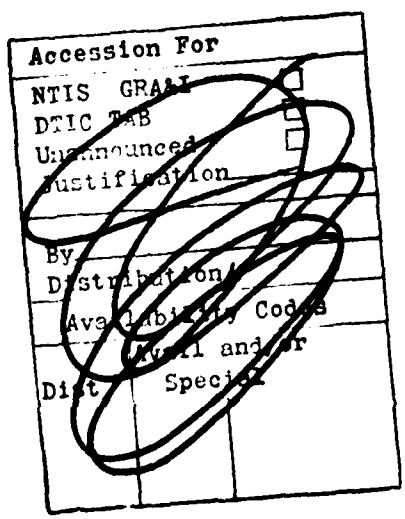

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RESULTS

During the first six months of this contract, Leishmania mexicana mexicana and L. brasiliensis panamensis served as standard, biochemically characterized cutaneous and mucocutaneous subspecies for drug screening. In general, observations in vivo on these two models for screening may briefly summarized as follows:

1. Both amastigotes and promastigotes (primary or subcultured) may be used to yield palpable lesions for screening.
   a. Promastigotes grown in several insect media (Figs. 1-3) are as infective as amastigotes for mice (Figs. 4).
   b. In one of these media, Schneider's + 20% HIFCS, there is no loss in infectivity through several subcultures (Fig. 5). Stationary phase cells are more infective in all media tested.

2. Rapid and reliable development of lesions for screening occurs when 0.1 ml is inoculated intradermally (ID) or subcutaneously (SC) at the tail base of mice (Figs. 6-9) as follows:

TIME (in weeks)

		Leishmania brasiliensis	L. mexicana
Amastigotes	(10^7)	3 - 5	2
Promastigotes	(10^8)	2 - 3	3
	(10^7)	3 - 6	4
	(10^5)	7 - 10	7

*Time to lesion formation is slightly longer if hamster to mouse inocula are used (Fig. 9).

Since promastigotes of these subspecies show excellent growth in Schneider's medium, are as infective as amastigotes regardless of age in culture or subculture, are the natural infective agent, and can be counted accurately to produce reliable lesions within a short time, we plan to use them to initiate infections.

Drug screening has chiefly consisted of establishing standard doses of Pentostam and Glucantime. The following is a brief summary of our results for L. m. mexicana and L. b. panamensis:

1. There is no effect of either Pentostam or Glucantime upon early or late amastigote or promastigote-initiated lesions for either subspecies when doses up to 233 mg/kg/day antimony x 5 are used (Figs. 6-9).

2. Both subspecies respond similarly to Pentostam, chronic infections with L. b. panamensis may be more sensitive to Glucantime during treatment (Fig. 9).

3. Although there is a temporary effect upon early amastigote-initiated infections, even after retreatment lesion size increases by week 9 to former levels (Figs. 7-9).

4. The SD50 and SD90 for these subspecies is well above that for visceral leishmaniasis (29 and 58 Sb^V mkd) in BALB/c mice (Table I), and appears to be approximately 461 and 800 Sb^V mkd, respectively (1-3, 9-10, these data).

In succeeding months, we plan to initiate drug tests using another L. m. mexicana (LTB 0016) and L. b. brasiliensis (M 1128). The decision to substitute these strains for those originally tested is based upon the following data accumulated during the first 6 months of this contract. Although L. m. mexicana WR 183 was initially isolated by P.C.C. Garnham in Panama and is considered a type specimen, this strain routinely visceralized in hamsters (JSK, unpublished observations) and BALB/c mice (these data). Therefore, we have selected another well-characterized strain of this subspecies which does not. Furthermore, although initial experiments using a well-characterized strain of L. b. brasiliensis from Tres Bracos, Brazil (LTB 0018) are still promising, we have yet to establish reliable lesions in large numbers of BALB/c mice. Therefore, we have substituted M 1128, another well-characterized strain from Belem, Brazil which grows well in vivo and in vitro.

In order to complete our roster of the most common South American cutaneous and mucocutaneous subspecies, we have also begun testing L. b. guyanensis (M 1142) and L. m. amazonensis (LV72 & 78; LTB 150492). Both of these subspecies are clinically and biochemically unique from their cohorts, and the latter has been extensively used in vitro for drug-screening. Apparently, L. b. guyanensis contains the highest amount of an unusual cyclopropane fatty acid (G.G. Holz, pers. comm.). This lipid relic from a predominantly prokaryotic and/or plant ancestry seems to be utilized efficiently (5) and may be an ideal target for chemotherapy in mucocutaneous species. Therefore, this subspecies merits special consideration. In summary, these mucocutaneous and cutaneous subspecies are currently being screened:

L. brasiliensis complex

L. b. brasiliensis (M 1128, LTB 0018)
L. b. guyanensis (M 1142)
L. b. panamensis (WR 120)

L. mexicana complex

L. m. amazonensis (LV 72 & 78, LTB 150492)
L. m. mexicana (LTB 0016)

LTB = Tres Bracos, Brazil
LV = Liverpool, England
M = Belem, Brazil

To augment ongoing studies by W.L. Hanson, we have initiated drug screening against L. donovani 1S (Sudan) in our mouse model. Results over the first six months may be summarized as follows:

1. Mice inoculated IC or IV with 10^7 splenic amastigotes or promastigotes yield highly reproducible results if amastigotes in liver impressions are enumerated two weeks after infection (Fig. 10). Seven day assays are not reliable for mice, although they are highly reliable for hamsters.

2. Peak numbers of parasites occur in livers of BALB/c mice 4 weeks after infection (Fig. 10). Since IC inoculation is faster, and just as reliable as IV injection, we prefer to use it. It also allows direct comparison with Hanson's hamster data.

3. In addition, to determining the SD90 for Pentostam in mice (Table 1), we have tested the polyamine inhibitor α- difluoromethylornithine (DFMO) against L. donovani (Table 2). Although DFMO is active against African trypanosomes, when given alone in a similar regime (1%) in drinking water, it was ineffective against leishmaniasis.

4. Currently, we are testing two WRAIR compounds (BJ 84232 and BJ 58410) against visceral leishmaniasis in BALB/c mice.

In addition to these screening data, we have some observations on the possible mode of action of Pentostam in vivo, which may help explain the regular recurrence of mucocutaneous and cutaneous lesions after treatment. Ultrastructural evidence obtained from L. mexicana 1156 infected hamsters (6) and recent

subcellular and biochemical data on L. m. mexicana amastigotes in vitro (D. Hart, pers. comm.), indicate that these subspecies may have unusual pathways in organelles sensitive to the drug. Unlike pentamidine-treated L. tropica (4), the ultrastructure of L. mexicana amastigotes in hamster nose dermis still showed intact nuclei and kinetoplasts 8 weeks after infection and treatment with Pentostam (6). However, a large population of microbodies normally seen within the cytoplasm of amastigotes from placebo-treated animals was completely absent. These data, together with those of Hart, who showed that:

1. Pentostam inhibits amastigote to promastigote transformation prior to cell division and during peak metabolism,
2. β-oxidation of fatty acids is an important energy-generating pathway for L. mexicana amastigotes,
3. The enzmes associated with this pathway are located in a P_2 small microsomal fraction (=glycosomal) of these cells,

suggest that although Pentostam disrupts one amastigote pathway, the nucleus and kinetoplast are still intact. Therefore, parasites which temporarily switch to a less efficient pathway can resume proliferation and can cause lesion regrowth once drug pressure is removed. That cutaneous and mucocutaneous species might be more resistant to this drug than L. donovani is not surprising, since there are remarkable metabolic differences and compartmentation of enzymes in various species of Leishmania (7, J. Decker-Jackson and D. Hart, pers. comm.).

In vitro models.

In addition to these data, we have recently initiated an in vitro system for continuously culturing L. donovani in a macrophage cell line (J774), initially obtained from a BALB/c reticulosarcoma (8), by weekly passing 3 ml containing ~15 x 10^6 cells into 30 ml fresh medium and incubating in 5% CO_2 at 35° C. Currently, L. donovani has been maintained in continuous culture for two months, and we are now defining the conditions necessary for maintaining L. b. panamensis and L. m. mexicana in this same system. We think this cell line will be a valuable correlate of our in vivo screening once active drugs have been identified. In this system, within one week, a newly seeded culture becomes 100% infected and cells are extremely easy to handle.

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Figure 1

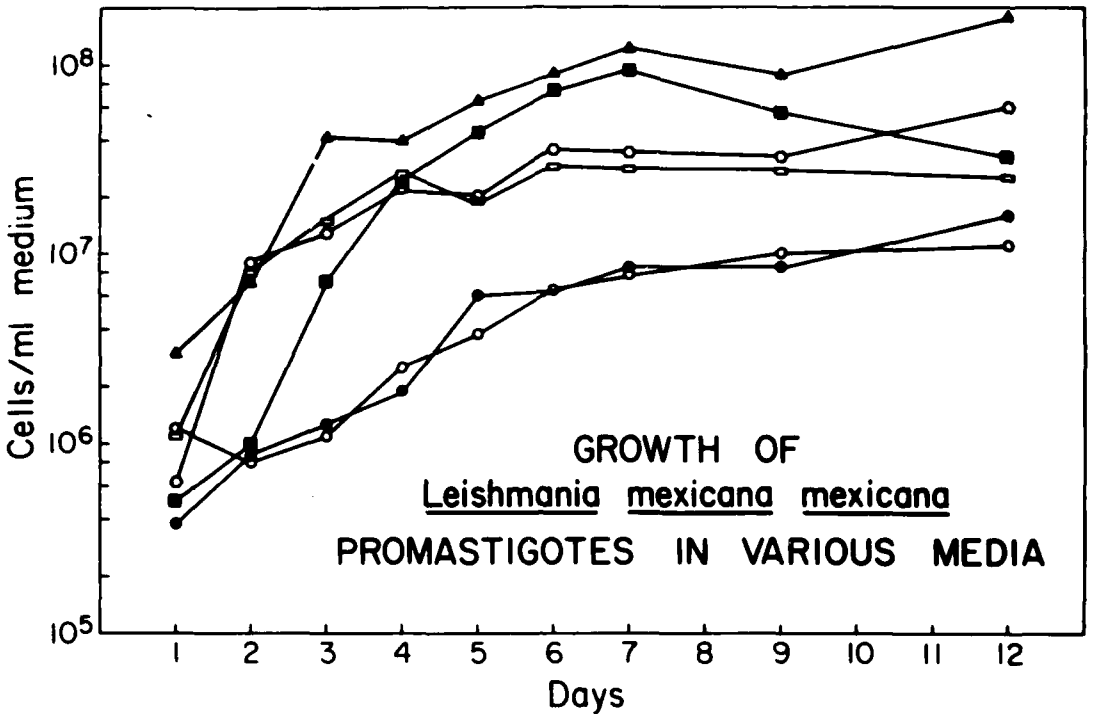

Figure 2

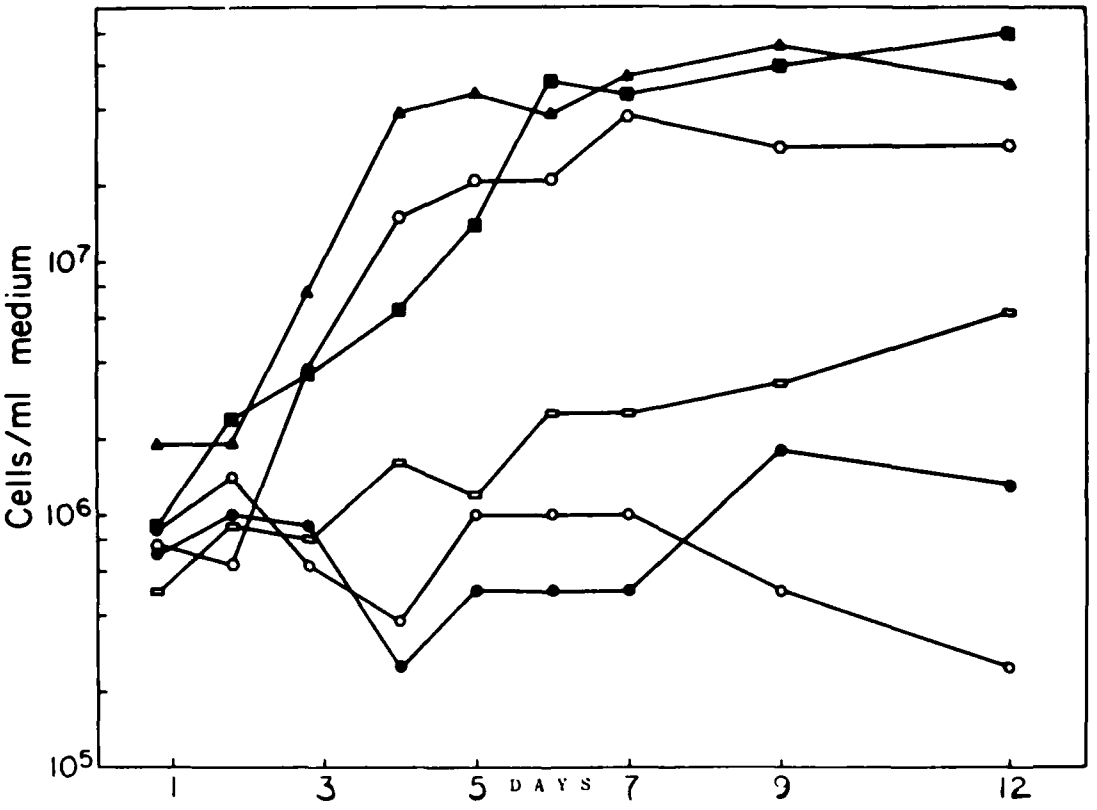

Figure 3

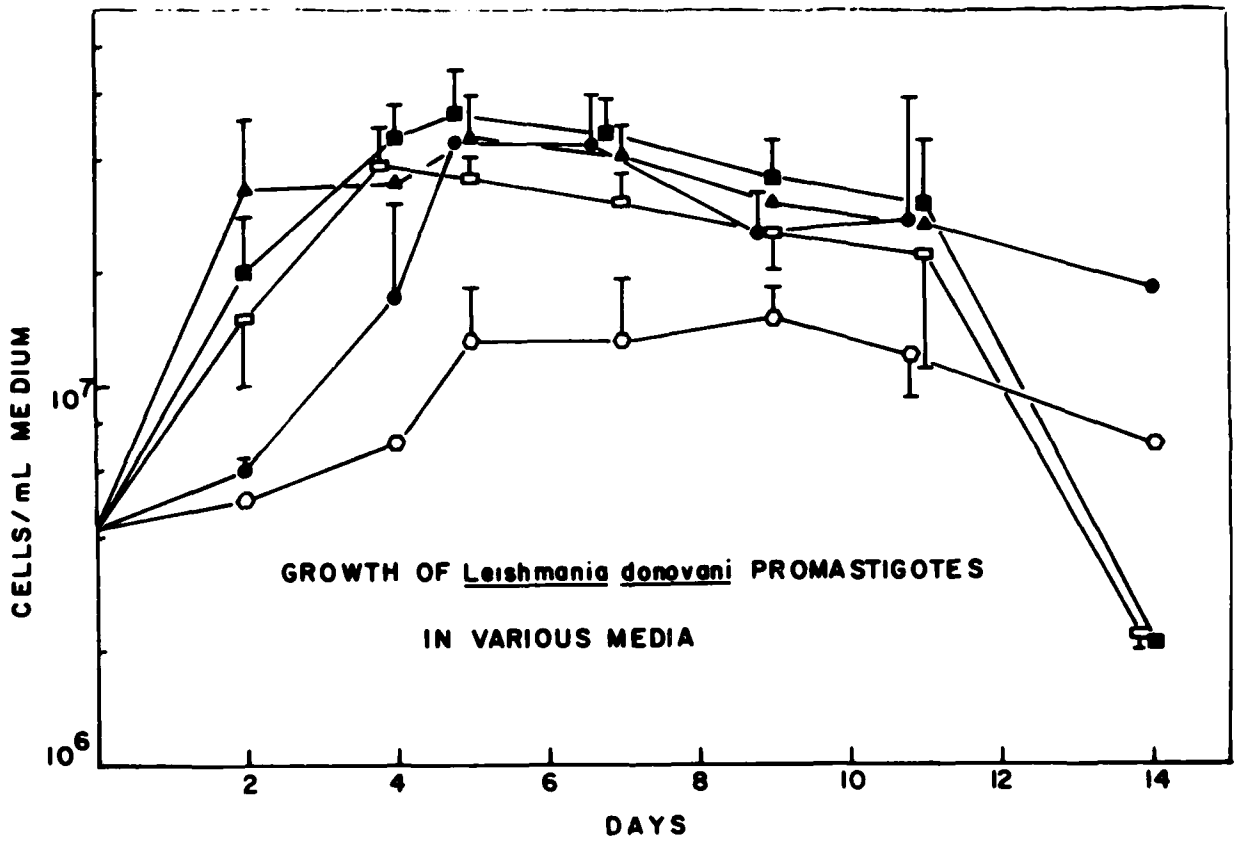
CELLS/mL MEDIUM
10^7
10^6
GROWTH OF Leishmania donovani PROMASTIGOTES
IN VARIOUS MEDIA
2
4
6
8
10
12
14
DAYS

Figure 4

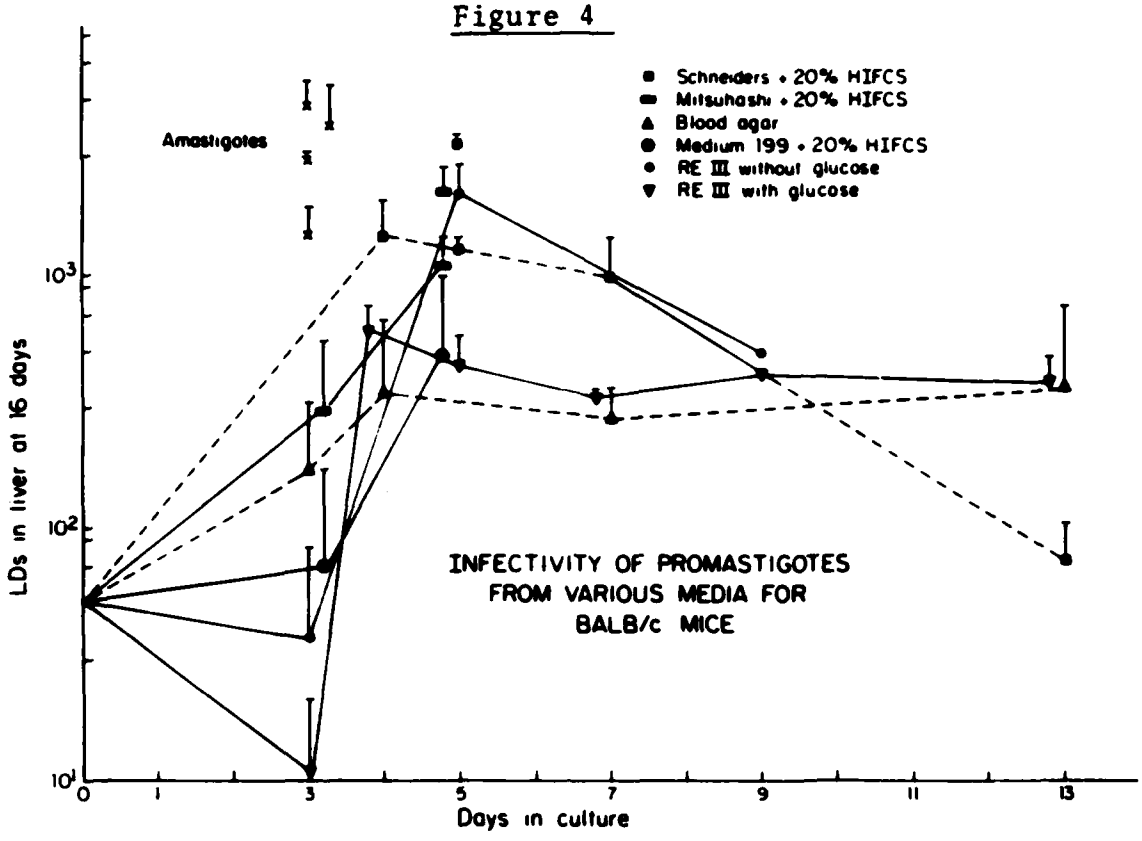

Figure 5

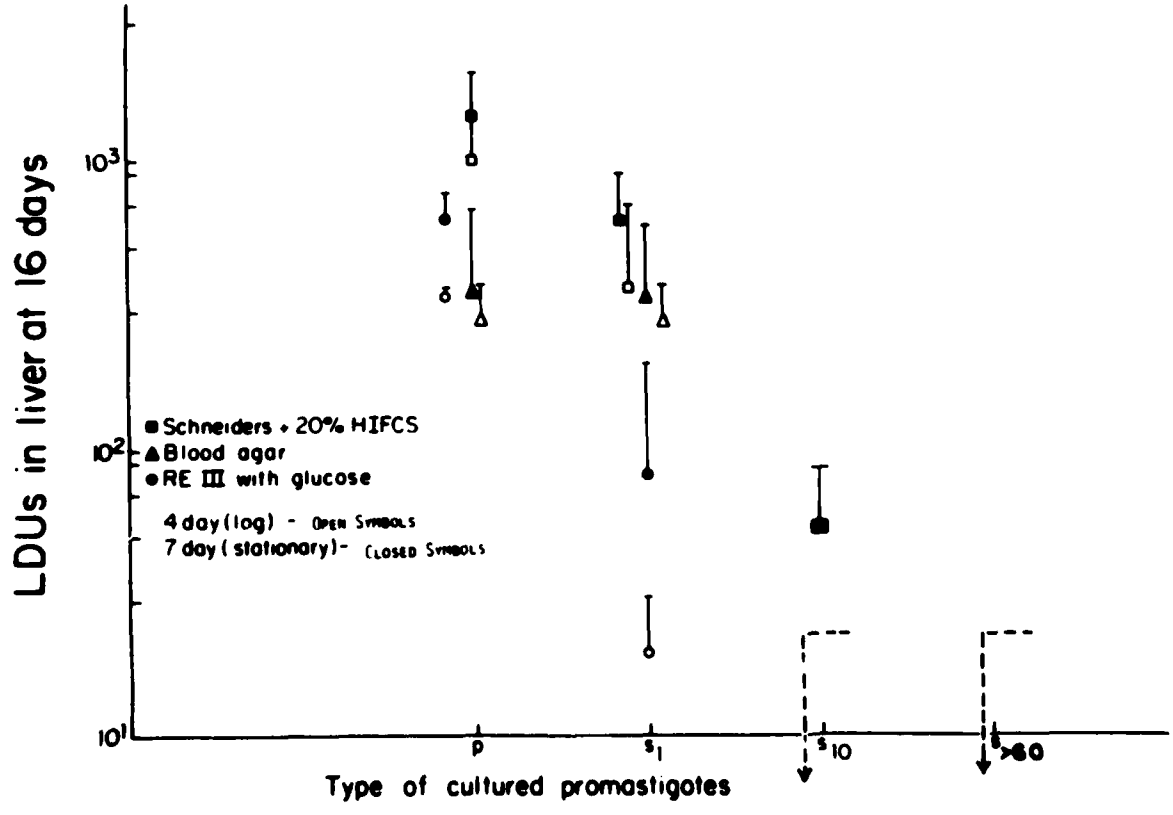

Figure 6

EFFECT OF PENTOSTAM ON *L mexicana mexicana* LESIONS IN BALB/c MICE

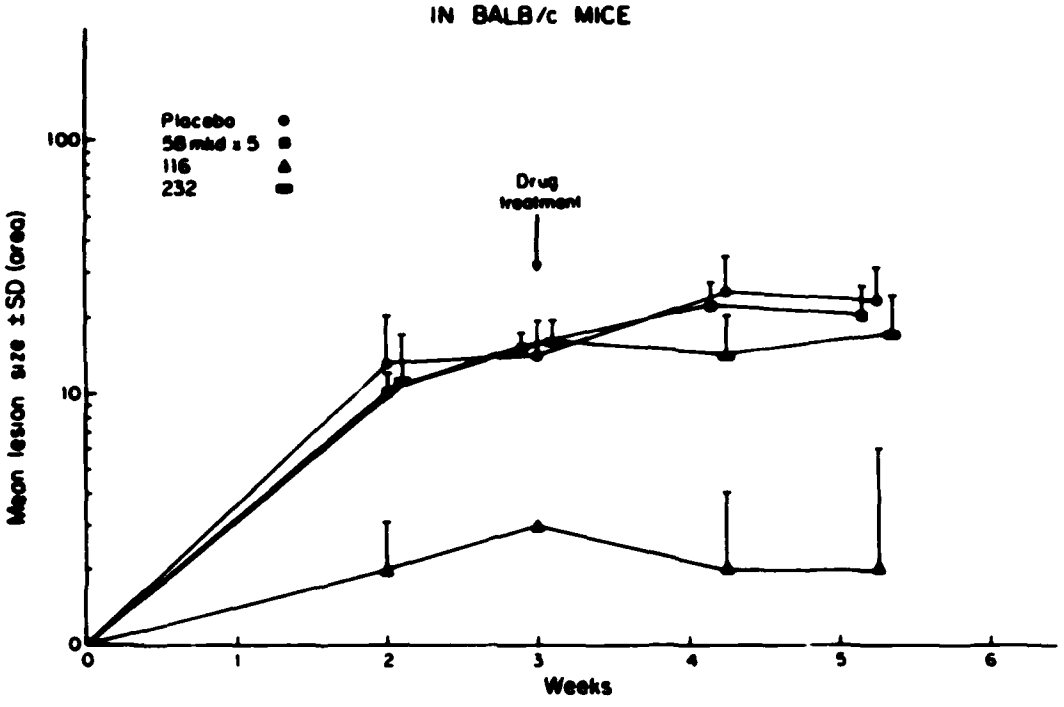

Figure 7

DEVELOPMENT OF *Leishmania m. mexicana* LESIONS IN BALB/c MICE
Single and Double Regimes of Sb^V

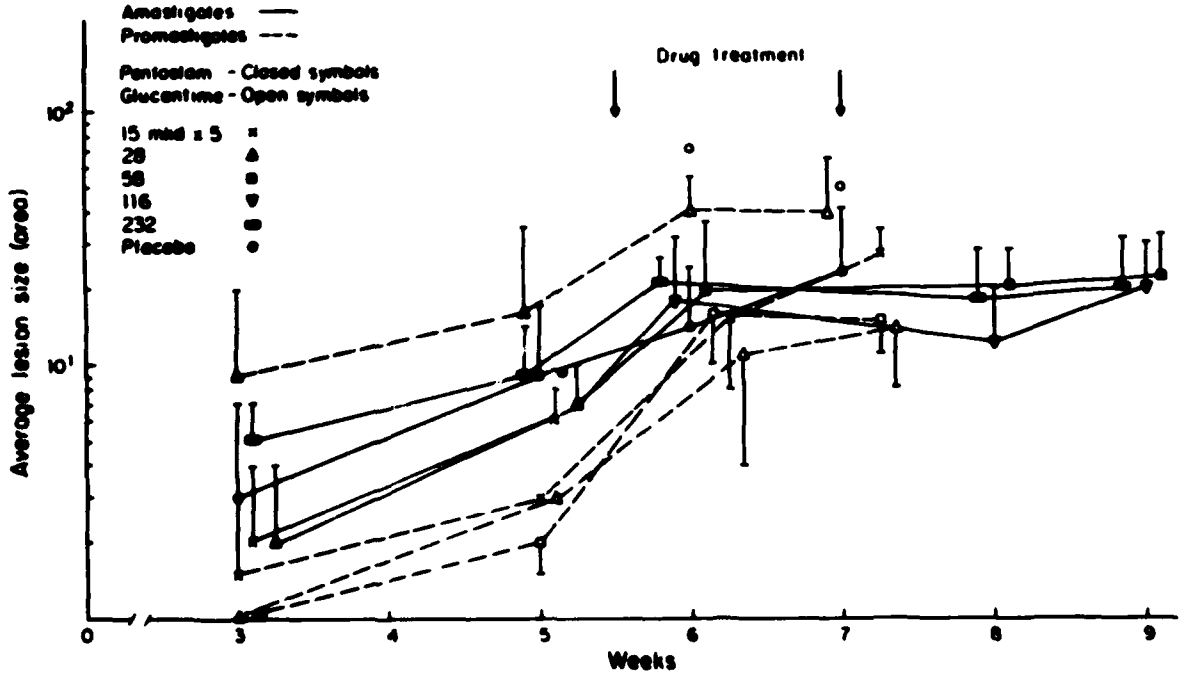

Figure 8

DEVELOPMENT OF *Leishmania brasiliensis panamensis* LESIONS IN BALB/c MICE
Single Regime with Sb^V

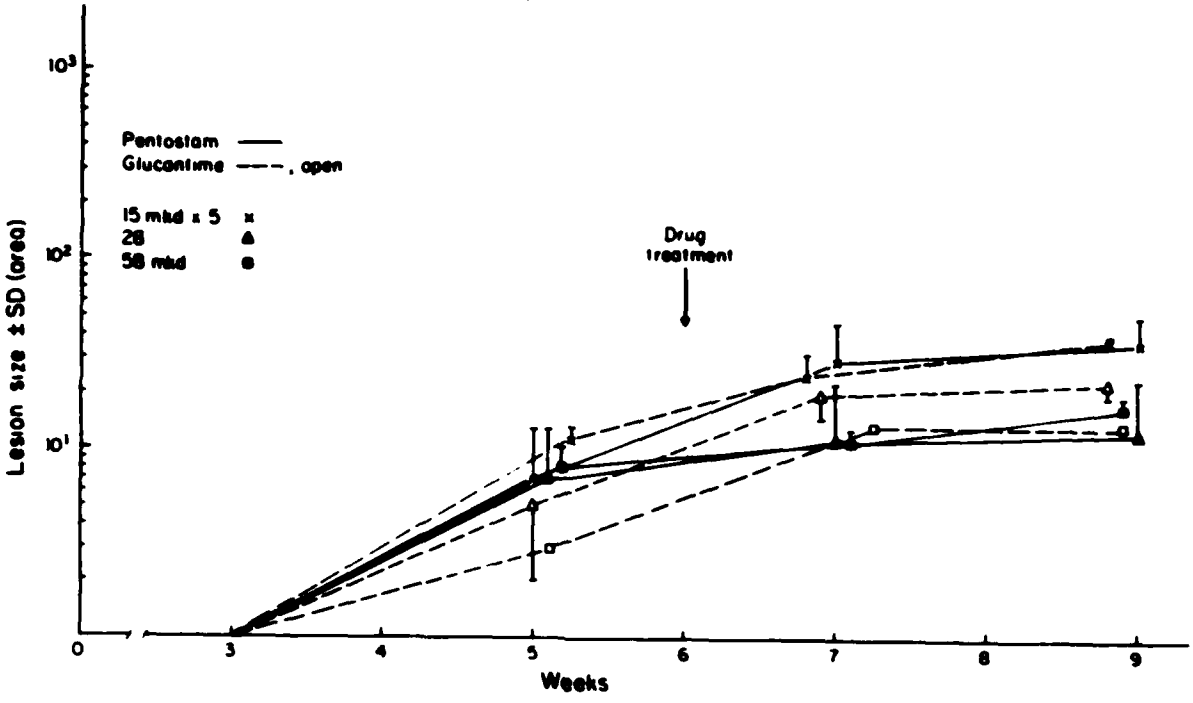

Figure 9

EFFECT OF Sb^V ON *Leishmania brasiliensis panamensis* AND *L mexicana mexicana* LESIONS IN BALB/c MICE

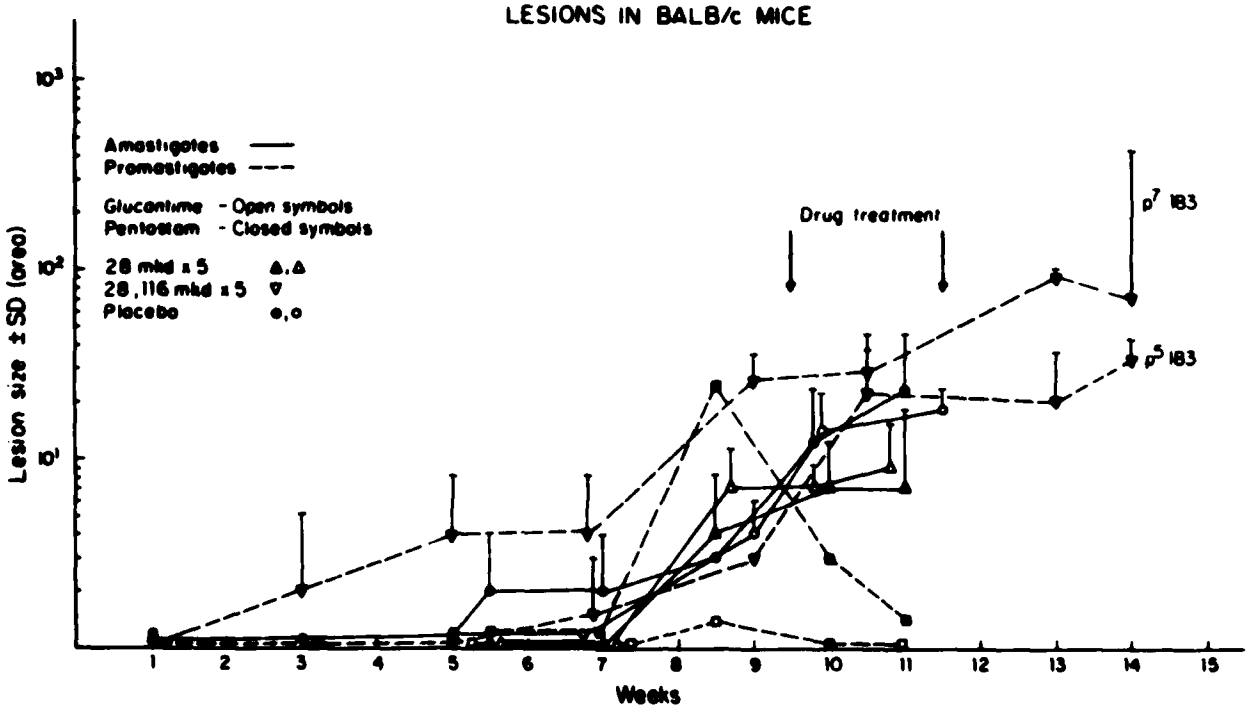

Figure 10

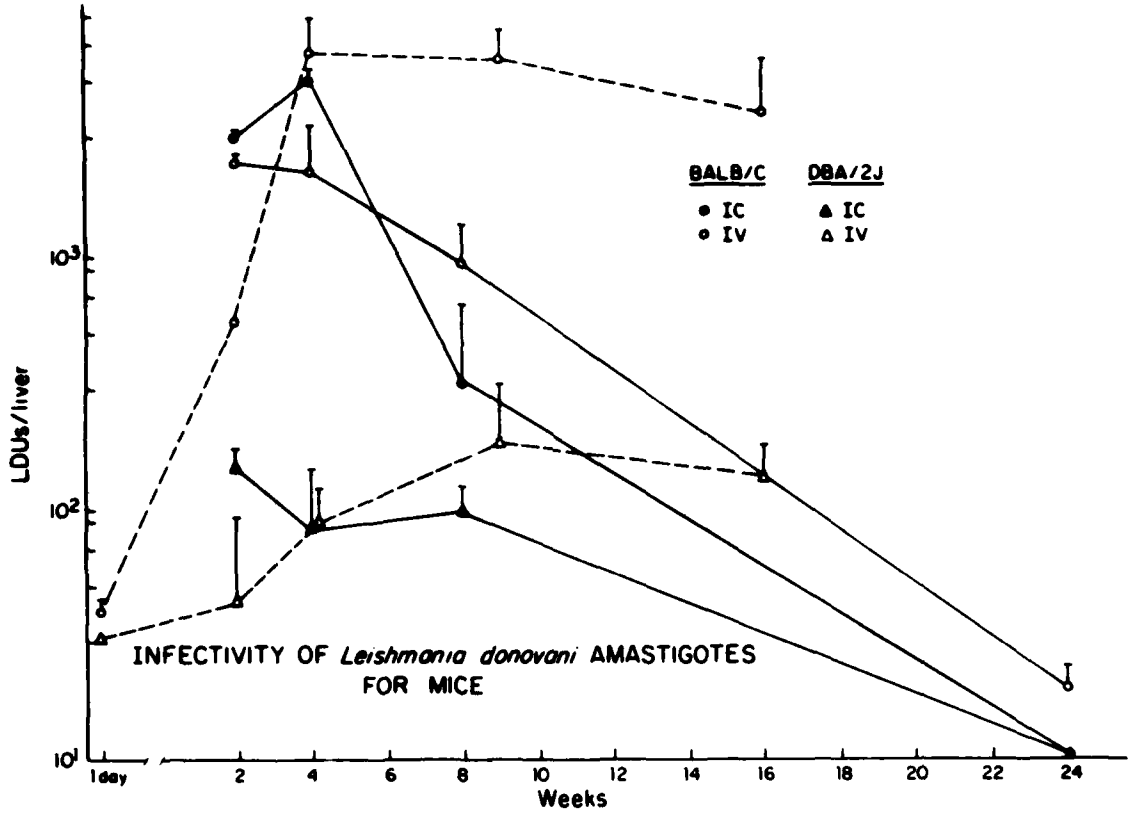
BALB/C
IC
IV
DBA/2J
IC
IV
LDUs/liver
10^3
10^2
10^1
INFECTIVITY OF *Leishmania donovani* AMASTIGOTES FOR MICE
1 day
2
4
6
8
10
12
14
16
18
20
22
24
Weeks

TABLE 1

Effect of Pentostam on Leishmania donovani in BALB/c Mice

Treatment	Amastigotes in Liver at 16 Days*
Placebo	2213 ± 424 (10)
Pentostam	
58	116 ± 115 (10)
104	3 ± 2 (10)
233	0 (10)

*Expressed as LDUs = amastigotes/liver cell nuclei/mg/liver

TABLE 2

Effect of RMI 71,782, an Inhibitor of Polyamine Biosynthesis on Leishmania donovani in BALB/c

Treatment	Number of Amastigotes in Liver at 16 Days
Placebo	$7 \pm 2 \times 10^7$ (5)
*RMI 71,782	
3 days pre-	$9 \pm 1 \times 10^7$ (5)
Time of infection	$7 \pm 6 \times 10^7$ (5)
3 days post-	$8 \pm 5 \times 10^7$ (5)
†Pentostam	$5 \pm 4 \times 10^6$ (5)

*200 mg/kg/day in drinking water for total of 10 days.
†13 mg/kg/day Sb^V IP x 5 days

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